

# Evidence That Barley 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase Kinase Is a Member of the Sucrose Nonfermenting-1-Related Protein Kinase Family<sup>1</sup>

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A protein kinase was partially purified from barley (*Hordeum vulgare* L. cv Sundance) endosperm by ammonium sulfate fractionation, followed by ion-exchange, Reactive Blue, Mono-Q, and phosphocellulose chromatography. It was shown to phosphorylate Arabidopsis 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and a synthetic peptide that was shown previously to act as a substrate for HMG-CoA reductase kinase purified from cauliflower, confirming it to be barley HMG-CoA reductase kinase. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the partially purified preparation showed the presence of a polypeptide with an approximate relative molecular weight ( $M_r$ ) of 60,000, which is the size predicted for the barley sucrose nonfermenting-1 (SNF1)-related protein kinases BKIN2 and BKIN12. Antisera were raised to a rye (*Secale cereale* L.) SNF1-related protein kinase (RKIN1) expressed in *Escherichia coli* as a fusion with maltose-binding protein and to a synthetic peptide with a sequence that is conserved in, and specific to, plant members of the SNF1-related protein kinase family. The maltose-binding protein-RKIN1 fusion protein antiserum recognized a doublet of polypeptides with an approximate  $M_r$  of 60,000 in crude endosperm extracts and a single polypeptide in root extracts, which co-migrated with the smaller polypeptide in the endosperm doublet. Both antisera recognized a polypeptide with an approximate  $M_r$  of 60,000 in the partially purified protein kinase preparation, suggesting strongly that barley HMG-CoA reductase kinase is a member of the SNF1-related protein kinase family.

Reversible protein phosphorylation is probably the single most important mechanism by which cellular activity is regulated in eukaryotes. The phosphorylation step is catalyzed by protein kinases, and the vertebrate genome may contain as many as 2000 protein kinase genes (Hunter, 1994), all of which can be considered members of one large gene family. These protein kinases regulate processes as

diverse as the response to extracellular messengers such as hormones, regulation of metabolism, commitment to cell division, and response to stress (Hardie, 1996). Although relatively few plant protein kinases have been characterized, it is reasonable to assume that they are as important in plants as they are in other eukaryotes.

One of the first protein kinases to be recognized was the mammalian AMPK. AMPK plays an important role in the regulation of lipid metabolism (Hardie, 1992; Hardie and MacKintosh, 1992; Hardie et al., 1989), inactivating by phosphorylation both acetyl-CoA carboxylase (Munday et al., 1988; Sim and Hardie, 1988; Davies et al., 1990, 1992) and HMG-CoA reductase (Clarke and Hardie, 1990). A convenient assay for activity of the enzyme has been developed using a synthetic peptide (the SAMS peptide) based on the primary phosphorylation site of rat acetyl-CoA carboxylase (Davies et al., 1989).

The catalytic subunit of AMPK is a  $M_r$  63,000 protein (termed the  $\alpha$  subunit) (Carling et al., 1989), and a full-length cDNA encoding AMPK- $\alpha$  from rat liver has been cloned and sequenced (Carling et al., 1994). The deduced amino acid sequence showed a striking similarity to the SNF1 protein kinase of *Saccharomyces cerevisiae* (Celenza and Carlson, 1986) and to a family of related plant protein kinases, including RKIN1 of rye (Alderson et al., 1991), BKIN2 and BKIN12 of barley (*Hordeum vulgare* L.; Halford et al., 1992; Hannappel et al., 1995), AKIN10 of Arabidopsis (Le Guen et al., 1992), and NPK5 of tobacco (Muranaka et al., 1994). The plant members of the family are all proteins with predicted  $M_r$ s of approximately 58,000, making them slightly smaller than AMPK- $\alpha$ ; however, SNF1 is larger, with an  $M_r$  of 72,000. However, the similarities between the sequences extend throughout their lengths, with amino acid sequence identities ranging from 44 to 46% overall and from 59 to 64% within the kinase domains. This discovery was unexpected because the function of SNF1 had appeared to be different from that of AMPK. Budding yeast uses fermentation of Glc preferentially for ATP production,

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Abbreviations: AMPK, AMP-activated protein kinase; HMG, 3-hydroxy-3-methylglutaryl; HRK-A, HMG-CoA reductase kinase-A; MPB, maltose-binding protein; SNF1, Suc nonfermenting-1.

and several genes encoding enzymes involved in other pathways of carbon metabolism are repressed when sufficient levels of Glc are present in the environment. This process is termed carbon catabolite repression (Gancedo, 1992) and involves a signal transduction pathway that links the perception of cellular Glc levels with the repression and derepression of Glc-repressible genes. SNF1 is integral to this pathway and Glc-repressible genes cannot be switched on in response to Glc deprivation in the absence of SNF1 activity (Celenza and Carlson, 1989).

Despite the different functions previously ascribed to AMPK and SNF1, the similarity in their amino acid sequences is compelling evidence that they and related plant protein kinases are homologous and are expected to have some functions and substrates in common. There is now considerable evidence to support this hypothesis. A functional relationship between SNF1 and RKIN1 from rye was demonstrated by expressing an *RKIN1* cDNA in a yeast *snf1* mutant strain (Alderson et al., 1991). SNF1 function was restored, allowing the yeast to grow on nonfermentable carbon sources. A similar result has since been achieved with the tobacco homolog NPK5 (Muranaka et al., 1994). Surprisingly, expression of AMPK in an *snf1* mutant did not complement the mutation (Woods et al., 1994), but biochemical evidence linking SNF1 and AMPK is now emerging. Yeast extracts contain an activity that phosphorylates the SAMS peptide, and this is absent in *snf1* mutant strains (Woods et al., 1994). Both SNF1 and AMPK will phosphorylate yeast acetyl-CoA carboxylase in vitro (Witters and Watts, 1990; Mitchelhill et al., 1994), and acetyl-CoA carboxylase activity has been shown to be regulated by SNF1 in vivo (Woods et al., 1994). However, there is no evidence that SNF1 is affected by AMP.

The biochemical evidence for the existence of a plant homolog of AMPK is equally strong. Two protein kinases (HRK-A and HMG-CoA reductase kinase-B) with similar biochemical properties to AMPK have been partially purified from cauliflower and other species (MacKintosh et al., 1992). They both phosphorylate the SAMS peptide and HMG-CoA reductase from animals and plants, although plant acetyl-CoA carboxylase does not appear to be a substrate and, like SNF1, neither kinase is activated by AMP. HRK-A, which accounts for approximately 90% of the SAMS peptide kinase activity, is an oligomer consisting of two or more subunits, but it has a catalytic subunit with an approximate  $M_r$  of 58,000 (Ball et al., 1994), which is the size predicted for the plant SNF1-related proteins.

A key question to be addressed is whether the plant SNF1-related protein kinases are equivalent to the catalytic subunit of the protein kinase purified from cauliflower and other species and identified as HRK-A (Ball et al., 1994). A considerable amount of information concerning the SNF1-related gene family in barley has already been obtained (Halford et al., 1992; Hannappel et al., 1995); therefore, barley endosperm was selected as the tissue for the present study. Two classes of SNF1-related transcripts are present in RNA isolated from barley endosperm, the *BKIN2* and *BKIN12* types. The former is also present in other organs, whereas the latter is unique to the seed (Hannappel et al., 1995).

In the present work we describe the partial purification of a protein kinase from barley endosperm using methods based on those described by Ball et al. (1994) for the purification of HRK-A from cauliflower, and we confirm that it is functionally related to HRK-A by showing that both the SAMS peptide and Arabidopsis HMG-CoA reductase will act as substrates in vitro. We demonstrate that antiserum raised against a fusion of MBP and RKIN1 expressed in *Escherichia coli* recognizes polypeptides of the expected size for BKIN2/BKIN12 in both crude endosperm and root extracts. Both this and an antiserum raised against a synthetic peptide with an amino acid sequence specific to plant SNF1-related protein kinases also recognize a polypeptide of the expected size in the partially purified protein kinase preparation.

## MATERIALS AND METHODS

Developing endosperm of field-grown barley (*Hordeum vulgare* L. cv Sundance) was harvested between 16 and 22 d postanthesis using a mechanical roller, immediately frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ .

### Purification of HRK-A from Barley Endosperm

All purification steps were carried out at  $4^{\circ}\text{C}$  using a procedure based on those described previously by Ball et al. (1994) and MacKintosh et al. (1992) for the isolation of HMG-CoA reductase kinase. Frozen endosperm (500 g) was powdered in a Waring blender with 8.35 g of Polyclar AT (Merck, Poole, Dorset, UK) and mixed with 500 mL of homogenization buffer (50 mM Tris-HCl, pH 8.2, 0.25 M mannitol, 50 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM benzamidine, and 0.1 mM PMSF). Triton X-100 was added to a final volume of 0.5% (v/v), and the homogenate was centrifuged (18,000g, 20 min). The supernatant was removed, and the pellet was re-extracted in an equal volume of homogenization buffer and centrifuged as described above. The combined supernatants were filtered through glass wool, and ammonium sulfate was added to 30% saturation. The suspension was stirred for 20 min and the precipitate was collected by centrifugation at 20,000g for 20 min. The pellet was redissolved in buffer A (50 mM Tris-HCl, pH 8.0, 50 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF, and 0.02% [v/v] Brij-35, and 10% [v/v] glycerol) and dialyzed in this buffer. The preparation was applied to a DEAE-Sepharose Fast-Flow ion-exchange column ( $5 \times 16$  cm,  $2 \text{ mL min}^{-1}$ , Sigma) equilibrated previously in buffer A. The column was washed with equilibration buffer until the  $A_{280}$  decreased to less than 0.15, and the kinase activity was then eluted using a linear gradient of 0 to 0.5 M NaCl in buffer A. Fractions containing activity were pooled and concentrated by the addition of ammonium sulfate to 60% saturation. After the preparation was stirred for 20 min, the precipitate was collected by centrifugation at 20,000g for 20 min, redissolved in 15 mL of buffer B (50 mM Tris-HCl, pH 7.2, 50 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF, 0.02% [v/v] Brij-35, and 10% [v/v] glycerol),

and dialyzed in this buffer. The dialyzed sample was applied to a Reactive Blue column ( $3 \times 6$  cm,  $0.5 \text{ mL min}^{-1}$ , Sigma) equilibrated in buffer B, and washed and eluted with buffer B containing  $0.5 \text{ M NaCl}$ . Fractions containing activity were pooled and applied to several NAP-25 columns (Pharmacia), which were equilibrated with buffer A. The desalted and equilibrated sample was then applied to a fast protein liquid chromatography Mono-Q HR 5/5 column ( $0.8 \text{ mL min}^{-1}$ , Pharmacia) equilibrated in buffer A and eluted with a linear gradient of  $0$  to  $1 \text{ M NaCl}$  in the same buffer. Active fractions were pooled and desalted on a NAP-5 column into buffer C ( $50 \text{ mM}$  Hepes [sodium salt],  $\text{pH } 7.8$ ,  $50 \text{ mM}$  sodium fluoride,  $1 \text{ mM}$  EDTA,  $1 \text{ mM}$  EGTA,  $1 \text{ mM}$  DTT,  $1 \text{ mM}$  benzamidine,  $0.1 \text{ mM}$  PMSF,  $0.02\%$  [v/v] Brij-35, and  $10\%$  [v/v] glycerol). The sample was layered onto a  $0.5\text{-mL}$  P11 phosphocellulose column (Whatman Biosystems) equilibrated in buffer C. The column was washed extensively in buffer C and then eluted with a stepwise gradient from  $0$  to  $1 \text{ M NaCl}$  in the same buffer. Fractions containing activity were concentrated using a Minicon-10 concentrator (Amicon, Lexington, MA), frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$  with no loss of activity.

#### Phosphorylation of the SAMS Peptide and Arabidopsis HMG-CoA Reductase

SAMS peptide kinase activity was measured using the standard assay described by Davies et al. (1989). Protein extract ( $5 \mu\text{L}$ ) was mixed with  $5 \mu\text{L}$  of kinase buffer ( $50 \text{ mM}$  Hepes,  $50 \text{ mM}$  sodium fluoride, and  $1 \text{ mM}$  DTT,  $\text{pH } 7.0$ ),  $5 \mu\text{L}$  of sterile water,  $5 \mu\text{L}$  of SAMS peptide stock solution ( $200 \mu\text{M}$ ), and  $5 \mu\text{L}$  of labeled ATP stock solution ( $1 \text{ mM}$  [ $\gamma\text{-}^{32}\text{P}$ ]ATP,  $25 \text{ mM}$  magnesium chloride). The reaction was incubated for  $30 \text{ min}$  at  $30^\circ\text{C}$ , and a  $15\text{-}\mu\text{L}$  portion was spotted onto a  $1\text{-cm}^2$  phosphocellulose P81 paper. The paper was immersed immediately in  $1\%$  (v/v) phosphoric acid to stop the reaction and washed for a further  $4 \text{ min}$ . The washing was repeated twice, and the paper was washed in acetone and dried on a heating block at  $100^\circ\text{C}$ . The paper was then transferred to a scintillation vial containing  $5 \text{ mL}$  of HiSafe Optisorb scintillation fluid (Wallac, Milton Keynes, UK) and counted in a liquid scintillation counter (Wallac). Specific activity was expressed as nanomoles of phosphate incorporated into peptide per minute per milligram of protein. All determinations were made in duplicate. Protein concentration was determined by the dye-binding method of Bradford (1976) with BSA as the standard.

HMGR1, an Arabidopsis HMG-CoA reductase (Enjuto et al., 1994) (kindly provided by Albert Ferrer, Barcelona, Spain), was used as a substrate for the partially purified protein kinase using the same procedure as for SAMS peptide phosphorylation, except that [ $\gamma\text{-}^{32}\text{P}$ ]ATP was present at  $6000 \text{ cpm pmol}^{-1}$  ATP (phosphorylation of mammalian HMG-CoA reductase by AMPK has been described previously by Carling et al. [1991] using this procedure, except that  $5'\text{-AMP}$  was added to the reaction in that case). The phosphorylated HMG-CoA reductase was precipitated using TCA with BSA as a carrier, and the

radioactivity in the pellet was determined by Cerenkov counting. The precipitated proteins were then resuspended and subjected to SDS-PAGE, and an autoradiograph was made of the gel immediately after staining.

#### Expression of Rye and Barley SNF1-Related Sequences in Escherichia coli

A  $1.8\text{-kb}$  *Stu*I/*Eco*RI restriction fragment of the rye *SNF1*-related cDNA from pcRKIN1 (Alderson et al., 1991) was cloned into pUC19 cut with *Sma*I and *Eco*RI to create pcRKIN1SE. pcRKIN1SE contains all of the coding region of the *RKIN1* cDNA and  $83$  nucleotides of the  $5'$  untranslated region. It was digested with *Bam*HI, and the resulting "sticky" ends were blunt-ended with Klenow fragment. The plasmid was cut again with *Eco*RI to release the insert, and this was cloned into pMAL-c (New England BioLabs) to create pMAL-RKIN1, which was then transformed into *E. coli* strain TB1 (*ara*/(*lac proAB*) *rpsL* ( $\phi 80 \text{ lacZ/M15}$ ) *hsdR*). pMAL-RKIN1 contains the *RKIN1* sequence downstream of and in frame with the *malE* gene, which encodes MBP (Duplay et al., 1984). Upon induction with  $3 \text{ mM}$  isopropylthiogalactoside, a fusion protein (Guan et al., 1987; Maina et al., 1988) of MBP and RKIN1 was expressed, and the cells were harvested, resuspended in lysis buffer ( $10 \text{ mM}$  sodium phosphate,  $30 \text{ mM}$  sodium chloride,  $0.25\%$  [v/v] Tween 20,  $10 \text{ mM}$  DTT,  $10 \text{ mM}$  EDTA, and  $10 \text{ mM}$  EGTA,  $\text{pH } 7.0$ ) and sheared in a BIOX X-press (Life Science Laboratories, Luton, UK). The fusion protein was dissolved in  $8 \text{ M}$  urea, the urea was removed by dialysis against lysis buffer, and the crude protein extract was applied to an amylose column (New England Biolabs). After the column was washed, the MBP-RKIN1 fusion protein was eluted by the addition of maltose. The eluted sample was then mixed with  $2\times$  SDS-PAGE loading buffer ( $1:1$ ) and electrophoresed on a  $10\%$  separating SDS-polyacrylamide gel. The fusion protein was recovered by electroelution (model 422 electroeluter, Bio-Rad) according to the manufacturer's instructions. The eluted protein was freeze-dried and used to prepare antiserum.

A  $633\text{-bp}$  fragment of the cBKIN12 cDNA (Halford et al., 1992) that encodes  $210$  amino acids at the C-terminal end of BKIN12 and a stop signal were cloned into pMAL-c to create pMAL-BKIN12. This was transformed into *E. coli* strain TB1, and a fusion protein of MBP and the C-terminal region of BKIN12 was expressed and found to be soluble. It was purified on an amylose column as described for MBP-RKIN1.

#### SDS-PAGE

Protein samples or crude endosperm extracts (prepared by grinding  $500 \text{ mg}$  of frozen barley endosperm in a mortar) were added to a microfuge tube containing an equal volume of  $2\times$  SDS-PAGE loading buffer ( $100 \text{ mM}$  Tris-HCl,  $\text{pH } 6.8$ ,  $200 \text{ mM}$  DTT,  $4\%$  SDS,  $20\%$  glycerol, and  $0.2\%$  bromophenol blue [Sigma]) and incubated at  $65^\circ\text{C}$  for  $10 \text{ min}$ . The solid material was pelleted, and  $10 \mu\text{L}$  of the supernatant was loaded onto a  $10\%$  SDS-polyacrylamide gel (Sambrook et al., 1989) for electrophoresis. Gels were

stained with Coomassie brilliant blue R-250 (Sigma) (10 mg in 100 mL of water containing 10% methanol and 10% acetic acid).

#### Amino Acid Sequence Comparisons

Plant SNF1-related protein kinase sequences were aligned and displayed using the PileUp and Prettybox programs, respectively, and a similarity search of the SwissProt database was performed using the Fasta program (Genetics Computer Group, 1991).

#### Peptide Synthesis and Preparation of Peptide Conjugate

Linear, unblocked peptides were synthesized by the University of Bristol Molecular Recognition Centre (UK) using 9-fluorenylmethyloxycarbonyl chemistry and coupled to BSA using 1-ethyl-3(3-dimethyl-aminopropyl)carbodiimide-HCl. Protein (10 mg) and peptide (10 mg) were dissolved in 1.5 mL of 10 mM sodium phosphate buffer, pH 6.5, at 4°C. 1-Ethyl-3(3-dimethyl-aminopropyl)carbodiimide-HCl (10 mg) was added, and the solution was stirred for 6 h, with the temperature rising to 20°C. The solution was dialyzed at 4°C against 1% (v/v) aqueous acetic acid and freeze-dried.

#### Preparation of Antisera

A New Zealand White rabbit was immunized intramuscularly with approximately 0.5 mL of saline solution containing the antigen emulsified in Freund's adjuvant. A booster injection was given after 28 d, and the first bleed was taken 14 d later. Subsequent bleeds were taken at 21-d intervals.

#### Immunodetection by Western Blotting

After separation by SDS-PAGE, proteins were transferred to nitrocellulose membranes (Amersham, UK) using a semi-dry blot system (Atto, Tokyo, Japan). Immunodetection was carried out according to the method of Johnson et al. (1984). Skim milk (10%) and 1% BSA in buffer (20 mM Tris, pH 7.5, 50 mM NaCl, and 0.05% Tween 20) were used for the blocking step and included in the first and second antibody incubations with the peptide antibody to reduce nonspecific binding. Immunoreactive bands were visualized using the horseradish peroxidase-based enhanced chemiluminescence system (ECL, Amersham). Manufacturer's instructions were followed throughout, except that crude antisera were used at titers of 1:500 and the secondary antibody (anti-rabbit immunoglobulin-horseradish peroxidase) at 1:1000.

### RESULTS

#### Purification of Barley Endosperm HMG-CoA Reductase Kinase and Phosphorylation of SAMS Peptide

Protein kinase activity was partially purified from barley (cv Sundance) endosperm harvested 16 to 22 d postanthesis using a procedure based on that described previously for the purification of HRK-A from cauliflower (Ball et al., 1994). This involved precipitation with 30% saturation

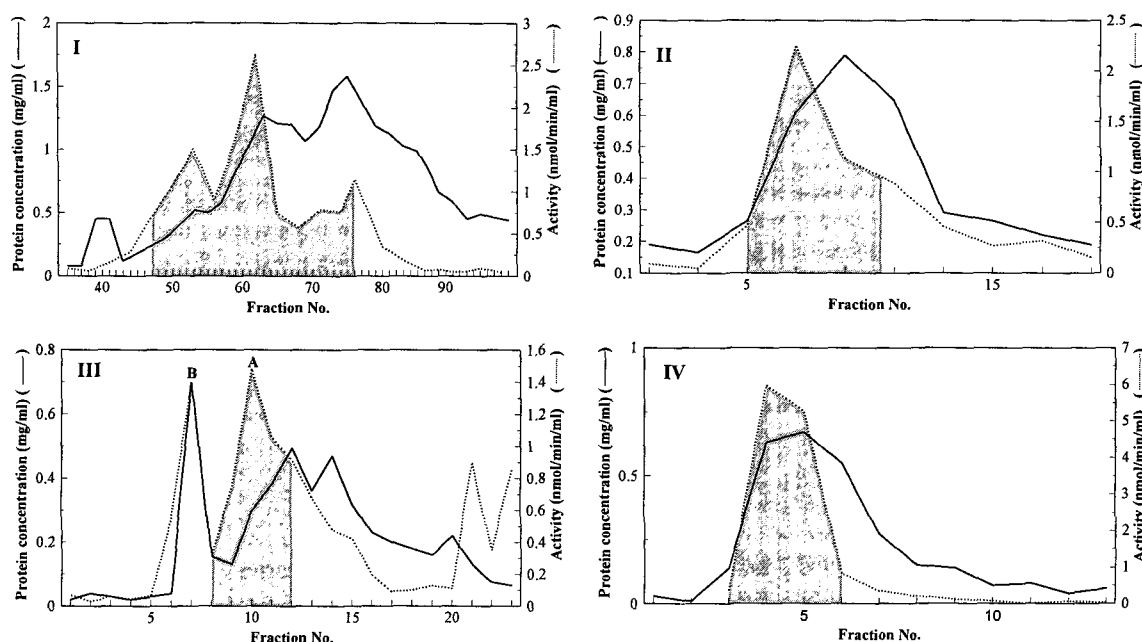
ammonium sulfate and ion-exchange chromatography (DEAE-Sepharose), followed by chromatography on Reactive Blue Sepharose, Mono-Q, and phosphocellulose. Through each purification step, peptide phosphorylation assays were carried out on the fractions using SAMS peptide (His-Met-Arg-Ser-Ala-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg) as a substrate (Davies et al., 1989). The sequence of this peptide is derived from the phosphorylation site of rat acetyl-CoA carboxylase, one of the substrates of the mammalian homolog, AMPK; it was shown previously to be phosphorylated by cauliflower HRK-A (Mackintosh et al., 1992). The activity profiles of the fractions eluted from each column and the fractions that were pooled and taken on to the next purification stage are shown in Figure 1. Two discrete peaks of activity (Fig. 1, peaks A and B) were present in the fractions that were collected from the Mono-Q column. The smaller peak B, which accounted for approximately 25% of the total activity, was not analyzed further in our study.

The specific activity of the kinase was 0.35 nmol min<sup>-1</sup> mg<sup>-1</sup> in the crude endosperm extract and 2.2 nmol min<sup>-1</sup> mg<sup>-1</sup> in the 0 to 30% ammonium sulfate precipitate. This compares favorably with values of 0.6 to 1.7 nmol min<sup>-1</sup> mg<sup>-1</sup> in equivalent ammonium sulfate fractions from various organs of other plant species (MacKintosh et al., 1992). Unfortunately, the recovery of activity was low, and the final specific activity was 25 nmol min<sup>-1</sup> mg<sup>-1</sup>, representing a purification of only 70-fold from the crude endosperm extract. The reasons for this low recovery of activity are not clear, since both protein phosphatase and proteinase inhibitors were included in the purification buffers. Apart from minor peaks, which resolved on both DEAE-Sepharose and Mono-Q, no significant peaks of peptide kinase activity were observed on any of the columns apart from the major peaks that were pooled for further study (Fig. 1). We, therefore, believe we have purified the major SAMS peptide kinase, albeit in a form that has low intrinsic activity.

Analyses of the kinetic properties of the partially purified barley protein kinase showed them to be similar to those of cauliflower HRK-A, the  $K_m$  (SAMS peptide) being approximately 47  $\mu$ M for the barley protein kinase compared with 50  $\mu$ M for HRK-A (Ball et al., 1994). The rate of SAMS peptide phosphorylation at various concentrations of the peptide and a Lineweaver-Burk plot of the data are shown in Figure 2.

#### Phosphorylation of Arabidopsis HMG-CoA Reductase

Recombinant Arabidopsis HMG-CoA HMGR1 was subjected to phosphorylation *in vitro* by the partially purified protein kinase using the procedure described by Carling et al. (1991). Proteins were precipitated from the reaction mixture with BSA as a carrier, separated by SDS-PAGE, and autoradiographed (Fig. 3, lane 1). Control experiments were performed in which the HMG-CoA reductase or the protein kinase preparation were omitted (Fig. 3, lanes 2 and 3, respectively). A band corresponding to the HMG-CoA reductase was clearly visible on the autoradiograph (Fig. 3, lane 1), whereas no phosphorylated products were de-



**Figure 1.** Protein concentration and activity profiles of fractions collected after DEAE-Sepharose (I), Reactive Blue (II), Mono-Q (III), and phosphocellulose (IV) chromatography of barley endosperm extract. Activity is shown as nanomoles of phosphate incorporated per minute per milliliter in the standard assay (Davies et al., 1989). Pooled fractions are indicated by shading. Discrete peaks of activity in the fractions collected from the Mono-Q column are labeled A and B.

tected in the absence of the partially purified protein kinase or in the absence of the HMG-CoA reductase. The specific activity of the protein kinase preparation with respect to HMG-CoA reductase was  $2.2 \text{ nmol min}^{-1} \text{ mg}^{-1}$ ; there was no evidence of autophosphorylation. The ability of the protein kinase activity to phosphorylate both the SAMS peptide and HMG-CoA reductase confirmed that it was barley HMG-CoA reductase kinase.

#### Expression of Plant *SNF1*-Related Sequences in *E. coli*

*RKIN1* and *BKIN12* sequences were expressed in *E. coli* strain TB1 as fusions with MBP using the pMAL system. The MBP-*RKIN1* fusion protein contained 502 amino acid residues from *RKIN1* (the entire protein), plus 27 amino acid residues encoded by the untranslated *RKIN1* leader sequence and three amino acid residues encoded by a section of pUC polylinker, fused to MBP ( $M_r$  40,000), giving an expected  $M_r$  of 101,000. The MBP-*BKIN12* fusion protein contained the 210 carboxy-terminal amino acid residues of *BKIN12* (residues 304–513) fused to MBP, giving an expected  $M_r$  of 64,000. Expression of the fusion proteins was induced by addition of isopropylthiogalactoside and cells harvested after 3 h.

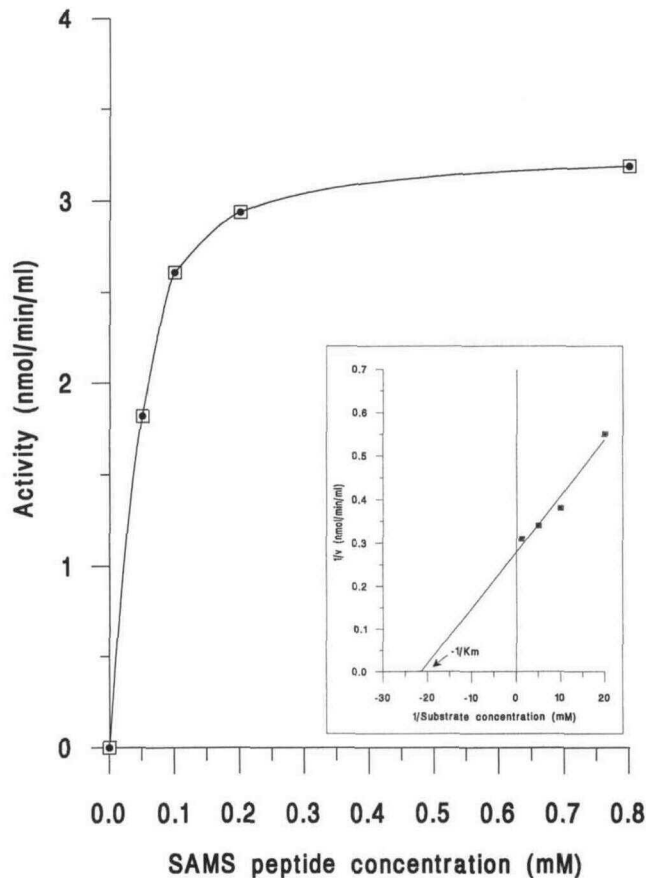
MBP-*RKIN1* was insoluble in the crude extract and could only be dissolved in 8 M urea, whereas MBP-*BKIN12* was soluble in the crude extract. The fusion proteins were purified by adsorption on an amylose column and were eluted by the addition of maltose. The only additional protein present after purification was MBP, which presumably resulted from instability of the chimeric gene or the fusion protein. A preparation of MBP-*RKIN1* was further

purified by eluting the polypeptide from an SDS-PAGE gel. This was used to prepare the antiserum.

MBP-*RKIN1* showed no activity in the SAMS peptide phosphorylation assay, and attempts to cleave the *RKIN1* protein from MBP failed, possibly because of the problems with solubility or inaccessibility of the proteolytic site. MBP-*BKIN12* was not tested for activity because it does not contain the catalytic domain of the protein kinase.

#### Synthesis of a Peptide with a Sequence Characteristic of Plant *SNF1*-Related Protein Kinases

Two *SNF1*-related protein kinase genes are expressed in the barley endosperm: *BKIN2* (Hannappel et al., 1995) and *BKIN12* (Halford et al., 1992). The derived amino acid sequences of the proteins that they encode were compared with those of *RKIN1* from rye (Alderson et al., 1991), *AKIN10* from Arabidopsis (Le Guen et al., 1992), and *NPK5* from tobacco (Muranaka et al., 1994) to identify sequences that are highly conserved within the plant *SNF1*-related family but not among other protein kinases. All eukaryotic protein kinases contain a catalytic domain of approximately 240 amino acid residues in which 11 conserved regions, separated by more variable regions, have been identified (Hanks et al., 1988). A strictly conserved sequence with the consensus Pro-Phe-Asp-Asp/Glu-Asn-Ile-Pro-Asn-Leu-Phe-Lys-Lys-Ile-Lys (residues 214–228 of *RKIN1*) is present between conserved domains IX and X of the plant *SNF1*-related protein kinases (Fig. 4). A peptide with this amino acid sequence was synthesized and termed the NIP peptide. It was coupled to BSA and used to produce antisera. Eleven of the 15 residues in this sequence are also conserved in yeast *SNF1*, whereas the second best



**Figure 2.** Rate of phosphorylation of the SAMS peptide ( $\text{nmol min}^{-1} \text{mL}^{-1}$ ) by the partially purified barley protein kinase at various concentrations of the peptide and Lineweaver-Burk plot (inset) of the data.

match-up identified in a search of the SwissProt database was found in PKC2, a protein kinase C homolog from *Drosophila* (Schaeffer et al., 1989), in which only five of the residues are conserved (Fig. 4). The only deviation from the consensus among the plant sequences occurs in BKIN12, where a Ser residue substitutes for the Asn residue at position 9 and an Arg residue substitutes for the Lys residue at position 12.

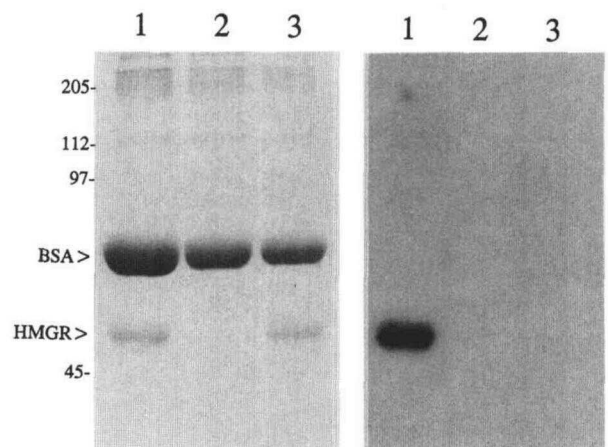
#### Western Blot Analyses

Western blot analyses of crude protein extracts from barley endosperm, roots, and leaves were carried out using the MBP-RKIN1 and NIP peptide antisera. The MBP-BKIN12 fusion protein was run alongside the extracts to act as a positive control. The NIP peptide antiserum did not prove useful in this experiment because of nonspecific binding (data not shown), but the MBP-RKIN1 antiserum clearly recognized a doublet of polypeptides with an  $M_r$  of approximately 60,000 in the endosperm extract and a single polypeptide in the root extract that co-migrated with the smaller polypeptide in the endosperm doublet (Fig. 5). None of these polypeptides were recognized by anti-MBP antiserum (data not shown).

Western blot analyses were also carried out on the partially purified HMG-CoA reductase kinase. SDS-PAGE analysis showed that the preparation contained several polypeptides, including one with an  $M_r$  of approximately 60,000 (Fig. 6A, arrow). The NIP peptide antiserum recognized the  $M_r$  60,000 polypeptide, although it also recognized at least three other polypeptides (Fig. 6B). Moreover, the  $M_r$  60,000 polypeptide was progressively enriched through the Mono-Q and phosphocellulose chromatography steps (Fig. 6B), and the cross-reaction was completely abolished by competition with the NIP peptide (data not shown). The MBP-RKIN1 antiserum was shown to react strongly with the  $M_r$  60,000 polypeptide (Fig. 6C) and very weakly with some  $M_r$  40,000 to 45,000 polypeptides, which may have been degradation products of the  $M_r$  60,000 polypeptide. The recognition of the  $M_r$  60,000 polypeptide by these two independent antisera raised to plant SNF1-related sequences is strong evidence that it is an SNF1-related protein kinase. The band was broad but did not resolve into a doublet as it did in the western blot of crude endosperm extracts.

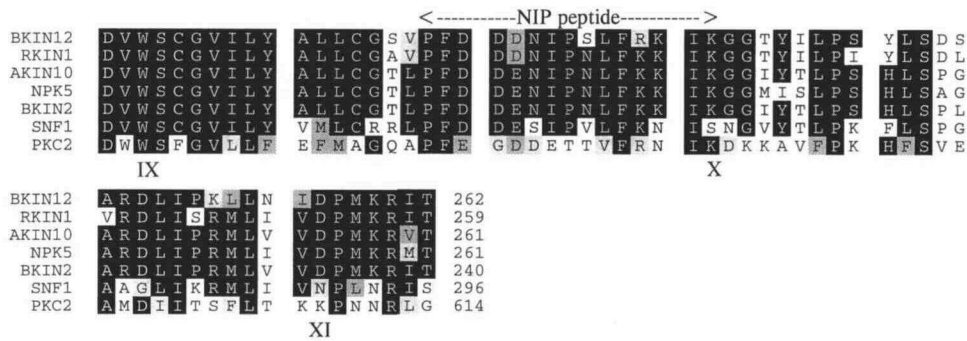
#### DISCUSSION

We have partially purified a protein kinase from developing barley endosperm and shown that it will phosphorylate HMG-CoA reductase and the SAMS peptide in vitro, confirming that it is the barley homolog of HRK-A, purified previously from cauliflower (MacKintosh et al., 1992; Ball et al., 1994). The specific activity for SAMS peptide phosphorylation in barley endosperm extracts after ammonium sulfate fractionation was slightly higher than that measured previously in other plant extracts (MacKintosh et al., 1992). However, the recovery of activity during purification was poor, despite the fact that the major peptide kinase peak was being pooled at each stage. It is not clear



**Figure 3.** Phosphorylation of HMG-CoA reductase by partially purified protein kinase. Lanes 1, Reaction mixture with Arabidopsis HMG-CoA reductase (HMGR) and protein kinase; lanes 2, HMG-CoA reductase omitted; lanes 3, protein kinase omitted. Coomassie blue-stained SDS gel is shown on the left, and an autoradiograph of the gel is shown on the right. The positions of size markers are indicated.





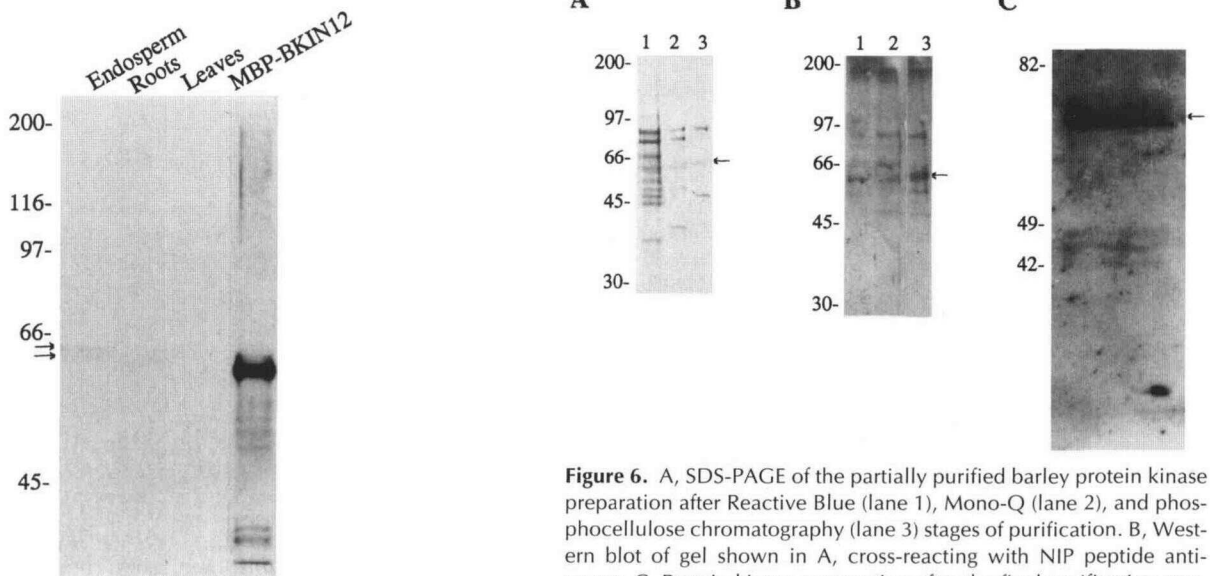
**Figure 4.** PileUp (Genetics Computer Group, 1991) alignment of AKIN10 from *Arabidopsis* (Le Guen et al., 1992), BKIN2 and BKIN12 from barley (Halford et al., 1992; Hannappel et al., 1995), NPK5 from tobacco (Muranaka et al., 1994), RKIN1 from rye (Alderson et al., 1991), SNF1 from yeast (Celenza and Carlson, 1986), and PKC2 from *Drosophila* (Schaeffer et al., 1989) showing the region from subdomain IX to subdomain XI of the catalytic domain (Hanks et al., 1988). The sequence chosen for the NIP peptide is indicated.

why this problem was much more severe in barley than in cauliflower, but, unless it can be resolved, barley will not be the material of choice for the purification of this enzyme, despite the high initial activity.

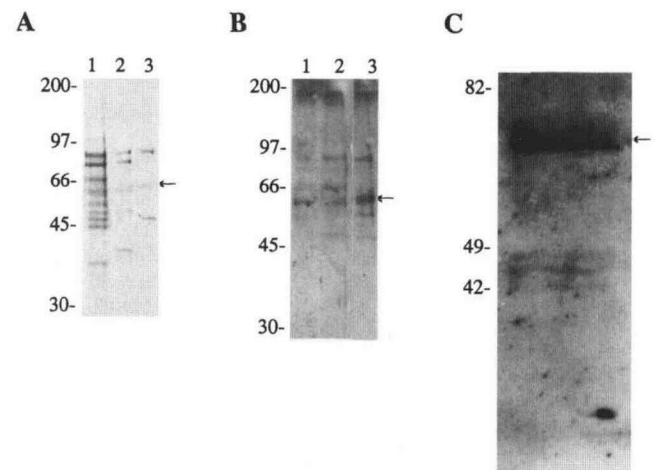
We have also presented convincing evidence supporting the hypothesis that the barley HMG-CoA reductase kinase, which we have partially purified, is a member of the SNF1-related protein kinase family. Barley SNF1-related protein kinases BKIN2 and BKIN12 (Halford et al., 1992; Hannappel et al., 1995) have predicted  $M_r$ s of 58,000, and the partially purified HMG-CoA reductase kinase preparation contains a polypeptide with an  $M_r$  of approximately 60,000. This polypeptide is recognized by two independent antisera, one raised to the rye SNF1-related protein kinase (RKIN1) expressed in *E. coli* as a fusion with MBP, and the other raised to a synthetic peptide (NIP) based on a sequence that is conserved in plant SNF1-related protein

kinases, but is not present in other protein kinases. The conclusion that plant HMG-CoA reductase kinases are members of the SNF1-related protein kinase family is further reinforced by the finding that cauliflower HRK-A is recognized by the antisera used in this study (Ball et al., 1995). It is also consistent with a previous observation that antisense expression of a potato SNF1-related sequence in transgenic potato tubers resulted in a reduction of up to 90% in SAMS peptide kinase activity in crude extracts (Halford et al., 1994).

There are 10 to 20 members of the SNF1-related gene family in barley (Halford et al., 1992) that can be divided into the BKIN2 and the BKIN12 types, both of which are expressed in the seed (Hannappel et al., 1995). BKIN2 and BKIN12 encode very similar proteins (68% sequence identity), and it is unlikely that the purification procedure



**Figure 5.** Western blots of crude barley protein extracts cross-reacted with MBP-RKIN1 antiserum. The positions of size markers are indicated. Cross-reacting polypeptides are indicated with arrows.



**Figure 6.** A, SDS-PAGE of the partially purified barley protein kinase preparation after Reactive Blue (lane 1), Mono-Q (lane 2), and phosphocellulose chromatography (lane 3) stages of purification. B, Western blot of gel shown in A, cross-reacting with NIP peptide antiserum. C, Protein kinase preparation after the final purification step, screened with MBP-RKIN1 antiserum. The positions of size markers are indicated. The  $M_r$  60,000 polypeptide, which cross-reacted with both antisera, is indicated with arrows.

would separate them or that the antibodies used in the present study would discriminate between them. Intriguingly, the band detected in the crude endosperm extracts by the MBP-RKIN1 antiserum resolved into a doublet, the larger band of which is not detected in root extracts; it is possible that the higher and lower bands represent the BKIN12 and BKIN2 forms, respectively. However, the doublet was not evident in the partially purified preparation, although the band recognized by the antiserum was broad.

The SNF1 family is a fascinating one, with roles in the regulation of a variety of fundamental cellular processes in eukaryotes. Undoubtedly, many of its targets and functions in the plant cell remain to be identified. However, the results presented here establish the barley SNF1-related protein kinases as one of the first plant protein kinase families for which nucleotide sequences have been determined, a purification procedure has been developed, and a substrate has been identified.

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